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Initial soil community drives heathland fungal community trajectory over multiple years through altered plant-soil interactions

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SUMMARY

- Dispersal limitation, biotic interactions and environmental filters interact to drive plant and fungal community assembly, but their combined effects are rarely investigated.
- This study examines how different heathland plant and fungal colonization scenarios realized via three biotic treatments - addition of mature heathland derived sod, addition of hay and no additions - affect soil fungal community development over six years along a manipulated pH gradient in a large-scale experiment starting from an agricultural, topsoil removed state.
- Our results show that both biotic and abiotic (pH) treatments had a persistent influence on the development of fungal communities, but that sod additions diminished the effect of abiotic treatments through time. Analysis of correlation networks between soil fungi and plants suggests that the reduced effect of pH in the sod treatment, where both soil and plant propagules were added, might be due to plant-fungal interactions since the sod additions caused stronger, more specific, and more consistent connections compared to no addition treatment.

- Based on these results, we suggest that the initial availability of heathland fungal and plant taxa, that reinforce each other, can significantly steer further fungal community development to an alternative configuration, overriding otherwise prominent effect of abiotic (pH) conditions.

Keywords: fungal community development, biotic interactions, soil pH, plant-fungal networks, heathland restoration, ITS1

INTRODUCTION

The incidence and abundance of local above- and below-ground species in an ecosystem are dependent on three main processes or ‘filters’: i) dispersal constraints ii) environmental (habitat) filters, and iii) biotic interactions (Belyea & Lancaster 1999; Lortie *et al.* 2004). Contrary to the traditional view that biotic interactions only operate after environmental filtering has taken place (Belyea & Lancaster, 1999; Raavel *et al.*, 2013), it is increasingly recognized that biotic interactions can significantly mediate species’ responses to the environment and therefore determine the strength and extent of this filter (Wisz *et al.*, 2013; Cadotte & Tucker, 2017; Aguilar-Trigueros *et al.*, 2017). The same is true for dispersal, where the timing of arrival may dictate which biotic interactions prevail, with a cascading effect on future community assembly through priority effects (Fukami *et al.*, 2005; Fukami, 2015). Understanding and predicting the development of communities thus requires knowledge of how these three processes act in concert (Wisz *et al.*, 2013).

Numerous studies have demonstrated that plant-soil interactions (particularly those between soil fungi and plants) are key biotic interactions that can shape above- and below-ground communities (Kardol *et al.*, 2006; Smith & Read, 2008; Wagg *et al.*, 2014; van der Putten, 2017). For instance, they have been shown to be major drivers of plant community composition patterns in restored tallgrass prairies (Bauer *et al.*, 2015) and pristine tropical forests (Mangan *et al.*, 2010). Moreover, manipulation through soil inoculation promoted the development of heathland and grassland systems, possibly through positive feedbacks among plants and their associated soil biota (Wubs *et al.*, 2016, 2019, van der Bij *et al.*, 2018). Studies investigating plant-soil interactions have particularly emphasised the

importance of mycorrhizal fungi as mediators between below- and aboveground communities (Bauer *et al.*, 2015) showing, e.g. that the presence and identity of mycorrhizal fungi determined whether late or early successional plant species came to dominate in a prairie restoration experiment (Koziol & Bever, 2017). Characterization of plant-soil interactions and the mechanisms by which they steer community assembly has been very challenging, particularly in field conditions, considering the myriad of interactions between plant and soil organisms (Toju *et al.*, 2018). Nevertheless, incorporating real-life complexity is crucial to accurately characterize the influence of the environment on plant-soil interactions (Lekberg *et al.*, 2018).

The complexity of plant-soil interaction can be captured by network approaches since they incorporate the whole community rather than limited number of preselected taxa (Ramirez *et al.* 2018; Toju *et al.* 2018). Several recent studies have utilized the network approach to examine putative biotic interactions (Banerjee *et al.*, 2016; Encinas-Viso *et al.*, 2016; Tylianakis *et al.*, 2018; de Vries *et al.*, 2018), showing for instance that the architecture of ecological networks is related to community stability (Thebault & Fontaine, 2010) and that hubs of highly connected soil microbes mediate interactions between plants and microbes (Agler *et al.*, 2016). Characterizing plant-soil network structure (e.g. the number and strength of connections) and identifying the taxa that are key players in these networks can thus help us understand how plant-soil interactions influence community development. Although correlation networks do not necessarily represent the real biological interactions between species, they can provide valuable insights in species co-occurrence patterns and elucidate the mechanisms driving their community assembly (Barberán *et al.*, 2012).

The present study examines the importance of plant-soil interactions for soil fungal community development in a large-scale heathland restoration experiment. Heathlands are species-poor systems thriving on nutrient-poor, acidic soils, with high dominance of ericaceous plants and associated ericoid mycorrhizal (ERM) fungi (Gimingham, 1989; Webb, 2008). Therefore, they represent a relatively tractable model system to explore typically complex plant-fungal interactions. In our study system, the upper soil layer from an ex-arable field was removed and different plots were subjected to three biotic addition treatments crossed with three pH manipulation treatments. Biotic treatments

represent different dispersal scenarios (different timing of colonization): an initial presence of both soil and plant propagules derived from a heathland system, an initial presence of primarily plant propagules only, or "natural" colonization through gradual dispersal in the control. The abiotic – pH – treatments created a gradient with the potential to act as an environmental filter within each of the biotic treatments. pH is known to strongly influence the success of heathland restoration (Marrs *et al.*, 1998) since it affects the germination of heathland plants and the development of their interactions with ERM fungi (Diaz *et al.*, 2008). By censusing the plant and soil fungal community composition through time, we followed the development of plant-fungal correlation networks under different treatments.

This experimental setup, therefore, allowed us to investigate the combined effect of three different mechanisms (timing of colonization, abiotic conditions, biotic interactions) on the development of soil fungal communities over multiple years. We hypothesized that (1) initial biotic manipulations had a lasting effect on fungal community development, as evidenced by significant differences in community composition at the end of the experiment; (2) that the effect of different biotic treatments and abiotic conditions were contingent on each other, as evidenced by interactions between biotic and abiotic treatments and variation in within-group dispersions between biotic treatments. Furthermore, we explored (3) whether and in what way the interactions between fungi or between plants and fungi may have contributed to fungal community development through co-occurrence and network analyses. Together, these approaches shed light on the relative importance and interaction between the ecological filters operating in heathland fungal community assembly.

MATERIALS AND METHODS

Study sites and sampling

Study sites were located at Dwingelderveld National Park (lat: 52.7810, long: 6.3709, alt: 10 m) in the Netherlands. The study area had previously been used for intensive agriculture. In 2011, the top-soil layer (30-40 cm) was removed to eliminate the excess of nutrients and other legacies (e.g. seed bank)

of agricultural land as an attempt to restore a typically nutrient-poor heathland ecosystem. Subsequently, 27 large plots (15m x 15m) were established with nine different treatments, three biotic treatments crossed with three abiotic treatments, each in three replicates in a randomized block design. The biotic treatments included biotic control = no additions, addition of hay material or addition of sod material, from well-developed heathlands. The abiotic treatments consisted of: no additions = abiotic control, addition of dolomite $\text{CaMg}(\text{CO}_3)_2$ = liming, or addition of elemental S = acidification. The donor heathland sites for sod and hay material was a dry mature heathland dominated by *Calluna vulgaris* L, located 100 – 200 m from the experimental site. For all treatments, the material was added in late autumn 2011 (first abiotic then biotic additions), except for hay material which was not available in late autumn and was added in early autumn 2012. For the hay / sod treatment, 1 m² of fresh heathland hay / sod material (the vegetation and soil down to 5-6 cm depth) was added per 2 m² and 15 m² of experimental site, respectively. For the liming / acidification treatment 2 t of dolomite / 1.5 t of elemental sulphur were added per hectare of experimental site, respectively. None of these treatments significantly altered the amount of organic matter in the soil, and except for the abiotic treatments, none altered the soil chemistry (Van der Bij et al. 2018), including pH (Supporting information Fig. S1). Initially, liming increased soil pH by approximately 0.3-0.5 units and acidification decreased it by 0.3 units (averaged across biotic treatments). Six years after the additions, soil pH under different abiotic treatments still differed significantly (mean pH₂₀₁₇: control = 4.7, liming = 5.2, acidification = 4.5) (Supporting information Fig. S1).

Every year from 2012 to 2017, plant cover in the centre 10*10 m of each plot was estimated according to the Tansley scale, and three soil samples were taken at a depth of 0-5 cm from each of the 27 plots and pooled into one composite sample per plot for microbial analysis and measurements of soil pH. In addition, three soil samples were taken in three different well-developed (reference) heathland plots in the same area in 2017 and pooled in one sample per reference. Samples taken in the first five years were immediately air-dried, homogenized and kept under cool, dark and dry storage conditions before the DNA was isolated in 2017, while the samples from 2017 were immediately frozen, shortly after which DNA was isolated. Further tests indicated that storage conditions and storage time did not affect perceived variation in fungal community composition. See Supporting

information (Method S1, Fig. S2) for more details on additional tests and analyses concerning sample preservation.

Sample preparation and sequencing

DNA was isolated from 0.25-0.35 g of soil using the DNeasy PowerSoil Kit according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). The ITS1 region was amplified using fungal primers ITS1f (Gardes & Bruns, 1993) and ITS2 (White *et al.*, 1990), modified according to (Smith & Peay, 2014). In the first PCR, primers were amended with Illumina Nextera labels (Illumina Inc; San Diego, CA, USA). Each 25 µl reaction mixture contained 2 µl of the sample, 0.5 µM of each forward and reverse primer, 1X PCR buffer, 200 µM dNTPs and 1 U Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). PCR conditions were as follows: initial denaturation at 98°C for 60 s, followed by 35 cycles of: denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; and an additional extension of 72 °C for 10 min. A second PCR was performed using dual barcoded primers with Illumina adapters (2.5 µl of 50 x diluted PCR products template and 0.1 µM of each primer). The conditions were: 98°C for 60 s, 12 cycles: at 98 °C for 10 s, 63 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. PCR products were run on an agarose gel to confirm successful PCR amplification and successful amplicons were normalized and purified from primers and primer-dimers using the SequalPrep Normalization Plate Kit (ThermoFisher Scientific). Samples were then pooled into a single library, and subjected to a gel extraction using QIAquick Gel Extraction Kit (Qiagen, Venlo, the Netherlands). The library was quantified with qPCR (KAPA Library Quantification Kits, Kapa Biosystems, Wilmington, MA, USA) and sequenced on the Illumina MiSeq platform (Illumina Inc; San Diego, CA, USA) with 300 cycles for forward and reverse reads. Several negative controls and technical replicates were also sequenced in order to test the reproducibility of sample preparation and the sequencing procedure (Supporting Information, Fig. S3). The raw sequences were deposited in SRA-NCBI database under the accession number: PRJNA566105.

Quality filtering and bioinformatics analyses

Fungal sequences were analysed using the USEARCH (v8.1.1861) and VSEARCH (Rognes *et al.*, 2016) software following the UPARSE pipeline (Edgar, 2013). After trimming to 250 bp the paired-end reads were merged and primers were removed. This trim length was chosen because it was the optimal length for merging pairs by removing the low-quality bases at the end. Merged sequences were quality filtered using expected number of errors (E) as a measure of read quality, as implemented in UPARSE. We imposed a relatively stringent criterion of $E_{\max} = 0.5$, keeping the reads that have maximum 50% chance to contain one erroneous base (Edgar & Flyvbjerg, 2015), leaving 3.01 M sequences. During merging and quality filtering approximately 70 % of sequences were discarded, many of which were likely primer-dimer sequences. Following singleton removal the sequences were clustered into OTUs (operational taxonomic units) based on 97% similarity using the UPARSE-OTU algorithm (Edgar, 2013) which automatically detects and filters out chimeras with high efficiency. All original reads were mapped to the OTUs with an identity threshold of 0.97, yielding an OTU table with a total of 2,192 OTUs and 3.5 M reads. Using all original reads does not compromise quality of OTUs but allows sequences erroneously labelled as low-quality to be counted. Further steps were performed using R software (R Core Team, 2015). The number of reads per sample was rarefied to 1,275. This rarefaction depth was chosen because it included almost all samples (except for four which were omitted), and although it does not represent the entire diversity, rarefaction curves showed that the number of taxa was levelling off for most samples at this depth. We also calculated Chao coverage (*entropart* package (Marcon & Herault, 2015)) as an indication of the amount of unsampled taxa, which was the same for different biotic treatments (Supporting Information; Fig. S4, Table S1). Representative OTUs were aligned to the fungal sequences in the UNITE database (Kõljalg *et al.*, 2005) (release date 10.10.2017), using the NCBI's BLAST algorithm with default settings. OTUs were retained and assigned to particular taxa if they had a minimum alignment length of 75 bp. and a maximum E-value of 10^{-36} (as in Waring *et al.*, (2016)).

Statistical analyses

The differences in fungal community composition were examined with PERMANOVA analysis (Anderson, 2001) using *adonis* function in *vegan* (Oksanen & *et al.*, 2018), based on Bray-Curtis (BC) distances and visualized using Nonmetric multidimensional scaling (NMDS, *metaMDS* in *vegan*). First, PERMANOVA analysis was performed 1) on the entire dataset using year as a continuous variable and plot as strata to assess the effect of time and 2) using biotic and abiotic treatments and their interaction as explanatory variables and year as strata. In addition, a separate PERMANOVA analysis was performed for the last year of the experiment to assess whether the effect of different biotic and abiotic treatments was present at the end of the experiment. Data were log-transformed prior to analyses to reduce the impact of abundant taxa (Anderson *et al.*, 2006) which are typically overestimated due to the exponential nature of PCR, but the results were similar using different types of transformations (Supporting information, Table S2). To assess general trends in fungal OTU richness, the effect of time and different biotic and abiotic treatments (as well as their interactions) on fungal OTU richness was tested using the *lmer* function from *lme4* package with plot as a random effect.

Multivariate dispersion (distances from group-centroids) within different biotic treatments for each year was calculated using the *betadisper* function in the *vegan* package and by calculating the mean distance between each pair of samples within a treatment (using the actual BC distances between samples). Based on the results from *betadisper*, a posthoc test was performed to examine if dispersion was significantly different between different biotic treatments and P values were corrected for multiple testing (Benjamini & Hochberg, 1995). The rationale for this analysis is to explore whether there is fungal community convergence within biotic treatments (i.e. if the dispersion within treatment decreases), which we take as evidence that the relative influence of abiotics or random variation decreases. We also calculated the BC distances contrasting biotic treatments (sod vs control, hay vs control and sod vs hay) to visualize change through time.

We used dissimilarity overlap curve (DOC) analysis (Bashan *et al.*, 2016) to test whether the interactions between fungal taxa were important drivers of fungal community composition in different biotic treatments across all the years. Bashan *et al.* (2016) demonstrated that communities with high

overlap also become increasingly similar in abundance-patterns (so reduced dissimilarity) when their constituent taxa interact predictably. Following Bashan *et al.* (2016) and Verbruggen *et al.* (2018), a significant negative relationship between community overlap and dissimilarity of the 50% of data points with highest overlap was here taken as support that interactions between fungal taxa substantially influence fungal community composition. Null models were constructed to additionally confirm that no relationship was found in randomized data (see Bashan *et al.* (2016) for more details on the analysis).

DOC analysis was performed in MatLab v.9.0 (The MathWorks, Inc., Natick, Massachusetts, United States). All other analyses were performed in R (version 3.3.2) (R Core Team, 2015).

Network analysis

Numerous network analysis methods have been developed and used in different studies: from simple correlation-based methods (e.g. in Encinas-Viso *et al.* (2016) and de Vries *et al.* (2018)) to more complex methods such as hierarchical modelling of species communities (Ovaskainen *et al.*, 2017) and extended local similarity analysis (Xia *et al.*, 2011). Due to the specific nature of our data, we followed a procedure that first calculates a general relationship between taxa based on the full dataset, and then estimates the extent to which this relationship is realized in each sample. By first calculating the relationship between taxa in the full dataset we circumvent the problem of few replicates for each treatment-time combination and the issue of high within-group variance of fungal abundances and low within-group variance of plant cover data which would otherwise be very difficult to correlate. This is done by assigning higher weights to 1) better fit and 2) higher relative abundance / percentage cover compared to all other occurrences of the two queried taxa. This procedure is detailed below.

First, 65 dominant fungal OTUs (containing a minimum of 500 reads across samples) and 25 dominant plant species (occurring in more than 8% of plots) were selected and the Pearson correlations between taxa were calculated. Rare taxa were removed to reduce the effect of zero occurrences, but more than 60% of total plant cover/fungal sequences for each treatment/year were

included (Table S3). Correlations with Pearson r higher than 0.2 were further considered for the construction of correlation networks. We imposed this threshold as an initial filter against spurious correlations but set it low enough to account for inherent error due to low precision of actual plant cover estimates and noise due to random variation. A sensitivity analysis with different thresholds and different cut-offs of the number of OTUs and plant species showed that these alternative choices did not substantially influence overall network structure (Fig. S5). Next, a simple linear regression between each pair of fungal OTUs and plant species was performed to estimate the study-wide slopes and intercepts using ordinary least squares (OLS) regression. In order to estimate the realization of these relationships in different samples, the values for slopes and intercepts were then used to calculate the explained variation (EV) of the abundance of one taxon based on the abundance of the other for each sample in each year. More specifically, EV for a given pair of taxa (cases with double zeros were excluded) was calculated by subtracting the residual variation – RV (the difference between the actual abundance of a taxon (y) and the abundance predicted by the abundance of the other taxon (x) when using the slope (a) and intercept (b) as calculated above) from the total variation – TV (the difference in abundance of a taxon (y) and the mean abundance of that taxon (\bar{y}) across all the data) (Equation 1). This value was then multiplied by an index calculated as the square root of the product between the abundance of each taxon in a pair per plot per year, as a fraction of their maximum abundance in the dataset (x' and y') to obtain EV' (Equation 2). EV' was used as an indicator of connection strength. This means that the higher the abundances of both taxa relative to their maximum abundance, the score gets a higher weight. The reasoning behind this is that under lower abundances, which are less variable, the scores would be inherently higher than the scores at higher abundances (due to positive correlation between mean and variance). Finally, this calculation was performed for each year and obtained values were averaged: i) per biotic treatment and ii) per each combination of biotic and abiotic treatments. Negligibly low coefficients (<0.001) and those lower than zero were set to zero.

$$1) EV = \frac{TV - RV}{TV} = \frac{|y - \bar{y}| - |y - (ax + b)|}{|y - \bar{y}|}$$

$$2) EV' = EV \times \sqrt{\frac{x}{\max(x')} \times \frac{y}{\max(y')}}}$$

To further investigate the development of typical heathland community networks, all taxa were divided into two groups: i) heathland plants (i.e. *Calluna vulgaris* L., *Erica tetralix* L., *Rumex acetosella*, L., *Betula pendula* Roth, *Molinia caerulea* L., *Carex pilulifera* L. and *Juncus sp.*; often found in mature heathland vegetation), and heathland-related fungi belonging to the order Archaeorhizomycetales, Helotiales and the genus *Clavaria*, based on that they were found in high abundance in reference heathlands in the current study and/or that they are known to be abundant in heathlands (Englander & Hull 1980; Rosling et al. 2011) or to contain ERM fungal taxa (Zijlstra *et al.*, 2005); ii) non-heathland taxa including all other plant species and fungal taxa. The list of all plant species included in the network analysis is shown in the Supporting Information (Table S4).

The change in the total strength of heathland vs non-heathland links between plants and fungi over time (from 2013 to 2017) was plotted for biotic and abiotic treatments. The first year (2012) was not included since the hay treatment had only been established earlier that year. The links between fungi and plants in the early (2013) and the late phase of the experiment (2017) were visualized and overall network properties (number of connections, strength and modularity) were calculated. The strengths of links for individual taxa were normalized to a 0-1 range by dividing them with the highest overall strength value in the dataset. Weighted modularity was calculated based on the Walktrap algorithm (Pons & Latapy, 2005) which assesses the extent to which the network is divided into modules or clusters. It can range from -1 to 1, where positive values indicate that the number of edges within groups exceeds the number expected based on a randomly connected network, whereas higher values indicate stronger clustering (i.e. dense connections within and sparse connections between the clusters).

All calculations and network visualizations were performed in R using base functions and the *igraph* package.

RESULTS

Fungal community composition

Over the six years of the ecosystem development, there was a clear directional change in fungal community composition (Fig. 1) where time explained 12% of the variation ($F_{1,153} = 21.67$, $P = 0.001$). When controlling for the effect of time, both biotic and abiotic treatments significantly influenced the fungal community composition ($r^2 = 0.06$, $F_{2,146} = 4.92$ $P = 0.001$ and $r^2 = 0.05$, $F_{2,146} = 4.41$, $P = 0.001$, respectively) and there was a significant interaction between them ($r^2 = 0.04$, $F_{4,146} = 1.81$ $P = 0.001$). The direction of fungal community change was orthogonal to the reference heathlands community composition, indicating that overall community development across treatments was not directed towards the local reference communities (Fig. 1). In the reference heathlands, the most dominant orders were Archaeorhizomycetales and Helotiales comprising 57% and 15% of total reads, respectively. The relative abundance of these fungi consistently increased in experimental plots over time in all treatments (Supporting information, Fig. S6). This increase was fastest and reached the highest levels in the sod treatment where the sum of the relative abundances of Archaeorhizomycetales and Helotiales in 2017 was comparable to that in the reference heathlands (mean = 69%, sd = 16 vs mean = 72%, sd = 6, respectively).

In the last year of the experiment, both biotic and abiotic treatments still had a significant influence on fungal community composition ($P < 0.001$), with a slightly higher effect size of the former than the latter ($r^2 = 0.15$ and $r^2 = 0.13$, respectively), and a significant interaction between them ($r^2 = 0.17$, $P < 0.05$) (Fig. 2a). Within biotic treatments, both hay and sod treatments differed from the control ($r^2 = 0.11$, $P = 0.01$ and $r^2 = 0.14$, $P = 0.003$; respectively), to a similar extent as in previous years (see Fig. 3 for temporal development of between-treatment differences). In the case of abiotic treatments, fungal community composition significantly differed between the liming and the acidification treatment in 2017 ($r^2 = 0.12$, $P = 0.006$). The interaction between biotic and abiotic treatments is related to a larger response of fungal communities to abiotic treatments in the biotic control (grey symbols in Fig. 2a) than in the sod treatment; there was a steadily decreasing dispersion (dissimilarity

between samples across abiotic treatment levels) of fungal communities under sod treatment over time (Fig. 2b), that was significantly lower than that of the control communities in 2017 ($P_{adj.} < 0.05$).

Fungal OTU richness was also significantly affected by time ($F = 15.9$, $P < 0.001$), biotic treatments ($F = 6.4$, $P < 0.01$), interactions between biotic and abiotic treatment ($F = 3.3$, $P < 0.05$) and interaction between biotic treatment and time ($F = 5.9$, $P < 0.001$). OTU richness tended to decrease over time in all treatments (with high variation between replicate plots), and this decrease was the most prominent in the sod treatment, in that it had the highest mean richness in 2012 and the lowest in 2017 of all biotic treatments. The other significant effects (interaction between biotic and abiotic treatments, and biotic main effect) are more complex and not straightforward to discern (Supporting information, Table S5).

DOC analysis

We used DOC (dissimilarity overlap curve) analyses to test whether biotic interactions between fungal taxa were important factors in shaping their community composition for each biotic treatment. The results indicate that biotic interactions had a significant influence in shaping fungal community composition in the sod and the hay treatment, evidenced by a negative relationship between community overlap and dissimilarity at high overlap region (sod: slope = -0.24, $P_{real} = 0.005$, $P_{null} = 0.3$, hay: slope = -0.18, $P_{real} = 0.02$, $P_{null} = 0.8$). For the control treatment, there was no significant relationship between community overlap and dissimilarity (slope = -0.02, $P_{real} = 0.47$, $P_{null} = 0.7$) (Supporting information, Fig. S7).

Plant-fungal correlation networks

In 2013 (one year after all treatments were in place), the structure of plant-fungal correlation networks was very similar in the control and the hay treatment, consisting of relatively strong links between non-heathland taxa. In the sod treatment, however, the overall network strength was very low, with a

relatively high number of links (Fig. 4a). During the course of the experiment, the strength of links between heathland taxa increased while the strength of links between non-heathland taxa decreased, particularly in the hay and the sod treatment (Fig. 4b).

The increase in strength of heathland taxa links occurred in the early stages of development for the sod treatment and was consistent across each abiotic treatment (Fig. 4b). Furthermore, while the overall strength of connections increased by approximately 200%, the number of connections decreased by half (from 77 to 36). The core (most strongly connected) plant species was *C. vulgaris* with 12 links and a normalized strength of 1 (the highest strength for any taxon in any treatment). Modularity, which represents the extent of division of a network into modules or groups, decreased from 0.5 to 0.2 from 2013 to 2017. These results demonstrate that the taxa in the sod treatment became more interconnected over time, and the connections became stronger and more specific (i.e. occur almost exclusively between heathland taxa).

Overall network structure in the hay treatment in 2017 was similar to the one in the sod treatment, consisting primarily of strong links between heathland taxa (Fig. 4a) with *C. vulgaris* as a central species (12 links, strength 0.7). During previous years, the increase in heathland taxa in the hay treatment was 2-3 years delayed compared to the sod treatment and was altogether diminished in the liming treatment, where the strength of links between non-heathland taxa was still relatively high (Fig. 4b).

In the biotic control treatment, the increase in the strength of links between heathland taxa started only in 2016 and was weaker than in the two other treatments, particularly under liming conditions. Therefore, the network structure in 2017 (Fig. 4a) was still substantially different from the network structure in the sod and the hay treatments, with positive links both within heathland and non-heathland taxa (therefore higher modularity of the network = 0.5). Moreover, there were multiple core plant species; one from the heathland group – *C. vulgaris* with 7 connections (strength 0.4) and the other from the non-heathland group – *P. lanceolata* with 5 connections (strength 0.3).

Finally, given that most plant and fungal taxa in the network analysis occurred in all biotic treatments in 2013 at least once (Table S6), we expect there was no absolute dispersal limitation hindering the development of communities in the control treatment. Moreover, heathland taxa (plant and fungal) were present with similar frequencies in the control and the hay treatment at the beginning of the experiment (Fig. S8).

DISCUSSION

In the current study, we used a large-scale heathland restoration experiment to estimate the combined effects of different drivers of fungal community assembly. We found that 1) the initial presence of heathland soil communities and plant seeds had a persistent influence on fungal community composition and plant-fungal correlations networks after six years; 2) the early presence of the soil communities diminished the effect of abiotic (pH) conditions on both of these community aspects compared to the treatments without sod additions.

Timing of colonization alters the development of fungal communities – the role of biotic interactions

It has previously been shown that soil inoculation can significantly affect heathland community composition (Wubs *et al.*, 2016; van der Bij *et al.*, 2018), indicating that plant-soil biotic interactions are important in this ecosystem type. Here, we present three further lines of evidence to demonstrate the dynamic and nature of biotic interactions in the development of fungal community composition over a six-year time-scale. Firstly, there was a persistent difference in fungal community composition between biotic addition treatments and the control. This was true despite that biotic additions did not alter the initial soil abiotic conditions, and fungi could easily colonize the non-inoculated plots from the adjacent inoculated plots. Similar findings were reported by Wubs *et al.*, (2019), where single introductions of soil biota and plant seeds led to long-term legacies on the trajectory of community assembly. Secondly, the DOC analysis indicates consistent biotic interactions among fungal taxa under sod additions and to a lesser extent hay additions, but this signal was absent in control

communities. Thirdly, at the end of the experiment, the structure of plant-fungal correlation networks in the sod and in the hay treatment was clearly different from that in the control. In the first two treatments, the networks contained strong connections between "typical" heathland plant and fungal taxa whereas the control treatments exhibited relatively loose connections for either heathland and non-heathland taxa. Morriën *et al.* (2017) have previously shown that during the course of primary succession soil networks can become more tightly connected. Here, we show that after six years of development such connectivity is highly dependent on the initial biotic community, as only the networks formed under biotic additions become more strongly connected and more specific.

The importance of the initial presence of not only plant but also soil fungal partners is further corroborated by the slower development of links between heathland plants and fungi in the hay treatment compared to the sod treatment. Such dependence of plant community composition on soil biota is in line with many previous reports in greenhouse (van der Heijden *et al.*, 1998; Koziol & Bever, 2017) and field (Wubs *et al.*, 2019) settings. Specifically for heathlands, Van der Bij *et al.* (2017) found that typical heathland vegetation developed much faster and typical heathland plants reached a much higher cover when a heathland soil community was already present. Our results suggest that when heathland seeds are present from the beginning, but a matching soil fungal community is absent or present at low abundance, it is more difficult for heathland plants and their associated fungal communities to develop. Apparently, additional heathland-related fungi first have to disperse into the plots and become established, causing heathland plant-fungal links to develop later as compared to the sod treatment. However, once their abundance reaches a certain threshold, further development of the heathland system is relatively fast and ultimately resembles the sod treatment. This means that, in terms of heathland restoration, hay additions can in longer-term provide similarly successful results as sod additions.

In the control treatment, both plant seeds and soil microbes were introduced gradually through dispersal. These plots were situated next to the inoculated plots and close to a larger area of abundant heathland vegetation, which poses a significant source of heathland taxa available to colonize them. It has been shown that the vicinity of source sites is an important factor promoting heathland

community development (Torrez *et al.*, 2016; van der Bij *et al.*, 2017). Surprisingly though, despite the fact that control plots collectively contained the majority of plant and fungal taxa observed in other treatments, including heathland taxa, the increase in the strength of links between heathland plants and fungi was notably delayed or absent compared to the sod-inoculated plots. A small-scale mismatch between heathland plants and fungi in time and space is likely the reason that links between them are not often formed, leaving opportunities for non-heathland plants and fungi to establish. This could result in the local development of competing plant-microbe systems, as evidenced by higher network modularity in the control treatment; one consisting of heathland and the other of non-heathland plant and fungal taxa, with relatively weak positive links within these modules. Whether these links between plants and fungi are strong enough to fuel positive feedback will likely determine the long-term trajectory of the non-inoculated plots, and whether the heathland system can successfully be restored or an alternative one will eventually prevail. The stochastic processes operating in this heathland system are likely to contribute to the 50% of variance not accounted for by different biotic and abiotic treatments or time.

Together, these observations suggest that initial simultaneous presence of a relatively large pool of heathland fungi and plant seeds in the sod treatment promotes the early formation of strong positive plant-fungal feedbacks between heathland taxa, thus reinforcing their further development. These early feedbacks can create priority effects (Kardol *et al.* 2007) and hamper the successful development of non-heathland fungi, leading to lower overall OTU richness observed in the sod treatment. Mechanisms behind these feedbacks could be both symbiosis, such as between plants and mycorrhizal fungi (Kerley & Read, 1998) but also competition for limiting nutrients or direct antagonism between plants or fungi, as has been shown to elicit priority effects in nectar-yeasts (Vannette *et al.*, 2014; Fukami, 2015). That plant-fungal soil interactions have indeed a high potency in creating priority effects has previously been demonstrated by Peay (2018), where the timing of ectomycorrhizal inoculation had a strong effect on the development of pine seedlings and on their success against competitors associated with AMF.

Which fungi would be responsible for the differences between treatments and control? Members of two dominant fungal orders, Archaeorhizomycetales and Helotiales strongly increased under biotic additions, particularly in the sod treatment, where they reached an abundance similar to that in the reference heathlands. Therefore, even though soil communities in the experimental site did not move towards those in the reference in terms of OTU identities, they became similar in terms of dominant fungal groups, which might play similar roles in the ecosystem. It is well known that Helotiales contain taxa that are associated with heathland plants (Zijlstra *et al.*, 2005; Leopold, 2016). Archaeorhizomycetales are relatively poorly investigated fungi that are typically found in roots and rhizosphere (Rosling *et al.*, 2011, 2013) and might depend on root-derived carbon (Schadt *et al.*, 2003). Given that these fungi are very abundant in the reference heathlands, they potentially form important associations with heathland plants as symbionts or decomposers. Further research is needed to reveal more about the nature of connections of these fungi with heathland plants and their possible importance in heathland restoration.

Convergence of communities under sod additions – biotic interactions override the effect of pH

The factorial experiment with a crossed abiotic and biotic additions allows us to test whether this abiotic filter has precluded biotic interactions to play out, as a hierarchical model of community assembly would suggest (Belyea & Lancaster, 1999). Under this model, we should expect communities to increasingly sort according to the environmental gradient as species disperse in, where the biotic addition treatments are given a head start. In contrast, the multivariate dispersion analyses show that fungal communities in the sod treatment converge over time, regardless of abiotic differences. Furthermore, the plant-fungal correlation networks in this treatment were also not influenced by the differences in abiotic conditions. These results indicate that environmental and biotic filters interact with each other and do not influence heathland communities in a solely hierarchical way. In the absence of initial "target" soil communities, abiotic pressures were apparently more influential, and liming in particular favoured stronger positive links between non-heathland plants and fungi, which are typically generalist that are less successful on acidic soils. In contrast, the links between heathland taxa were promoted under acidification because heathland plants thrive under

acidic conditions (Lawson *et al.*, 2004; Diaz *et al.*, 2008, 2011) and likely heathland fungi too, as known to be the case for Helotiales (Rousk *et al.*, 2010).

This, however, raises the question of why the development of connections between heathland taxa in the sod treatment was not affected by sub-optimal (increased pH) conditions. It is possible that plant-associated heathland fungi can strengthen the heathland plant performance (and vice-versa) even under sub-optimal conditions through positive feedbacks, and hinder the establishment of other, otherwise competitively superior species that are developing in the control plots. Research on facilitation has highlighted that positive interactions between species - particularly mutualistic ones - can expand their tolerance to the abiotic environment (Callaway & Walker, 1997; Bruno *et al.*, 2003; Poisot *et al.*, 2011; Kazenel *et al.*, 2015; Peay, 2016; Gerz *et al.*, 2018). For instance, it has been shown that ectomycorrhizal fungal symbionts can help seedlings establish and persist under suboptimal conditions (Simard, 2009). Our results strongly suggest that, in heathland systems, biotic links can override "environmental filters" supporting the proposal of Cadotte & Tucker (2017) and Aguilar-Trigueros *et al.* (2017) that these are much less rigid than previously thought.

CONCLUSION

The findings presented here suggest that the timing of colonization has an important effect on the development of fungal community composition in heathland systems through shaping plant-fungal interaction networks. We propose that the early stage presence of heathland soil communities and the interactions they form can reinforce the development of a heathland system and alleviate the abiotic filter imposed in the absence of these interactions. If the system is exposed to slow dispersal, other incoming plant and fungal species establish their own, alternative interactions possibly leading to a strongly altered community trajectory that is more sensitive to the abiotic context. These results have clear implications for our capacity to steer community development, for instance in the context of heathland restoration, through manipulation of keystone plants and fungi.

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AUTHOR CONTRIBUTION

RB, MW, RvD, EV and DR designed the experiment and performed the fieldwork. DR performed the lab work, data analyses, data interpretation and wrote the first draft of the manuscript with the help of EV. MW, RvD, JH, MP and SV contributed to data interpretation and to the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. NMDS ordination showing the change in fungal community composition over the course of six years (from 2012 to 2017) compared to the reference heathland communities (ref). Different colours represent different years and dotted lines connect the samples from the same year with their group centroid. First two dimensions are shown (stress: 0.15). The ordination with the third dimension is presented in the Supporting information (Fig. S9).

Figure 2. a) NMDS ordination of fungal community composition throughout six years (2012 – 2017) where each year is presented separately to emphasize biotic and abiotic treatments. The first two dimensions are shown (stress: 0.15). The ordination with the third dimension is presented in the Supporting information (Fig. S10). Different colours represent biotic (control, hay, sod) and shapes abiotic treatments (control, acidification, liming). **b)** Bray-Curtis distance (dissimilarity) between each fungal community in a biotic treatment to any other sample from that treatment (i.e. dispersion within biotic treatments but across abiotic treatments) over the same six years as in (a). Values are slightly shifted to increase visibility.

Figure 3. Mean Bray-Curtis dissimilarity between fungal communities exposed to different biotic treatments through time. Different colours represent different combinations of biotic treatments (sod vs hay = grey, hay vs control = green, sod vs control = red). 75% percentiles are shown as error bars. If values decrease with time there is a tendency for fungal communities in treatment-pairs to become more similar, and vice-versa.

Figure 4. a) Positive plant-fungal interaction networks for 2013 and 2017 for three biotic treatments (control hay, sod). Green and red circles represent plant and fungal taxa, respectively. The size of the circles is proportional to percentage cover for plant species and relative abundance for fungal OTUs. Lines represent the edges (connections) between the taxa and their width is proportional to the strength of connections. Darker lines represent links between the heathland taxa and lighter represent links between other taxa (note: this includes the links between the pairs where one or both taxa were

classified as non-heathland and those that could not be classified). **b)** Change in the strength of links between heathland – H (full lines) and non-heathland – NH (dashed lines) taxa in time for control, hay and sod treatment. Different line colours represent abiotic treatments (grey – abiotic control, blue – liming, red – acidification). * = values higher than the maximum presented here are set to one for visibility.

The following Supporting Information is available for this article:

Fig. S1 Soil pH under different abiotic and biotic treatments from 2012 to 2017.

Fig. S2 Testing the effect of storage conditions.

Fig. S3 NMDS ordination showing the distance between technical replicates

Fig. S4 Rarefaction curves

Fig. S5 Sensitivity analysis for different cut-offs used in the network constructions

Fig. S6 Change in the relative abundance of dominant heathland taxa in time for three biotic treatments

Fig. S7 The results of DOC analysis

Fig. S8 Percentage of plots that contained heathland fungi and plant taxa for three different biotic treatments over time

Fig. S9 Change in fungal community composition with time (NMDS with first and third dimension).

Fig. S10 Change in fungal community composition with time shown for each year separately (NMDS with first and third dimension).

Table S1 The proportion of total diversity in a sample covered by the rarefaction threshold according to the Chao index.

Table S2 The results of PERMANOVA analyses using different types of transformations of OTU data

Table S3 Proportion of taxa included in the network analysis

Table S4 The list of plant species included in the network analysis

Table S5 Mean OTU richness for different treatments throughout the years

Table S6 The percentage of plant and fungal taxa present in the soils in different biotic treatments per year.

Methods S1 Testing the effect of storage conditions.

Figure 1

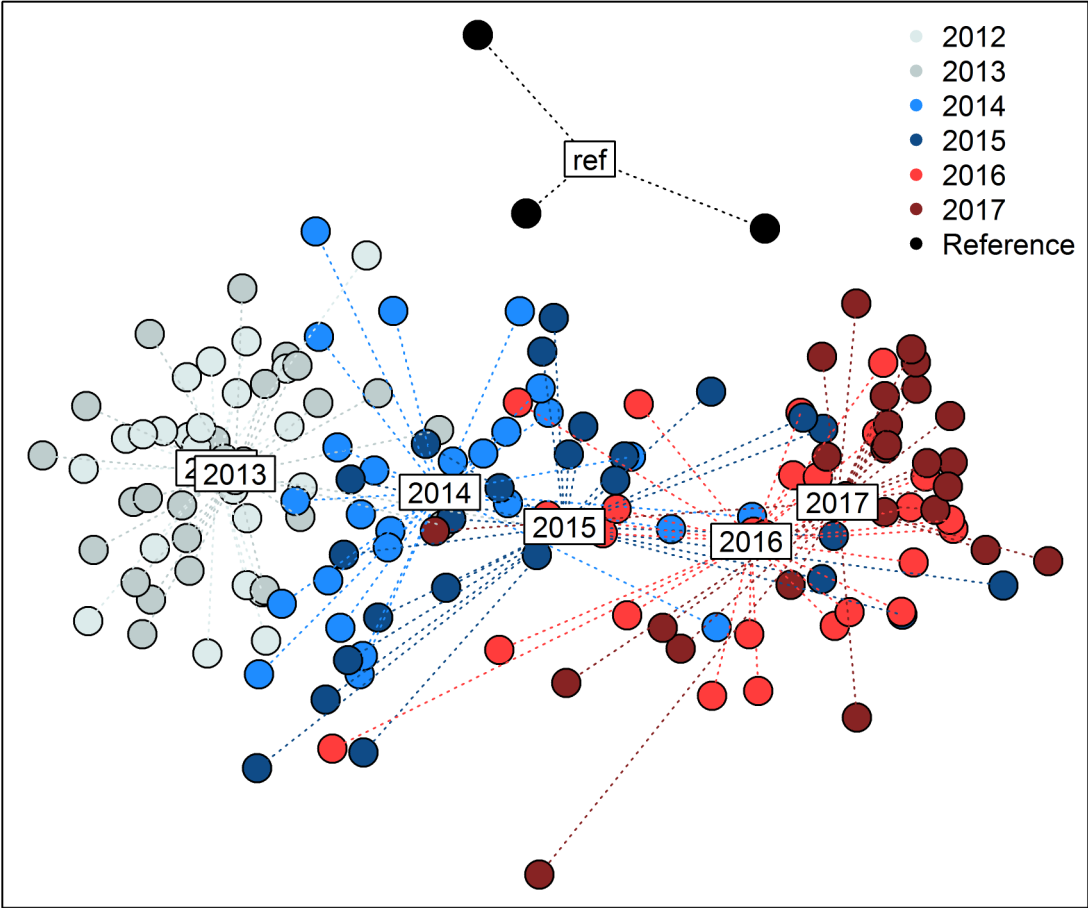


Figure 2

(a)



(b)

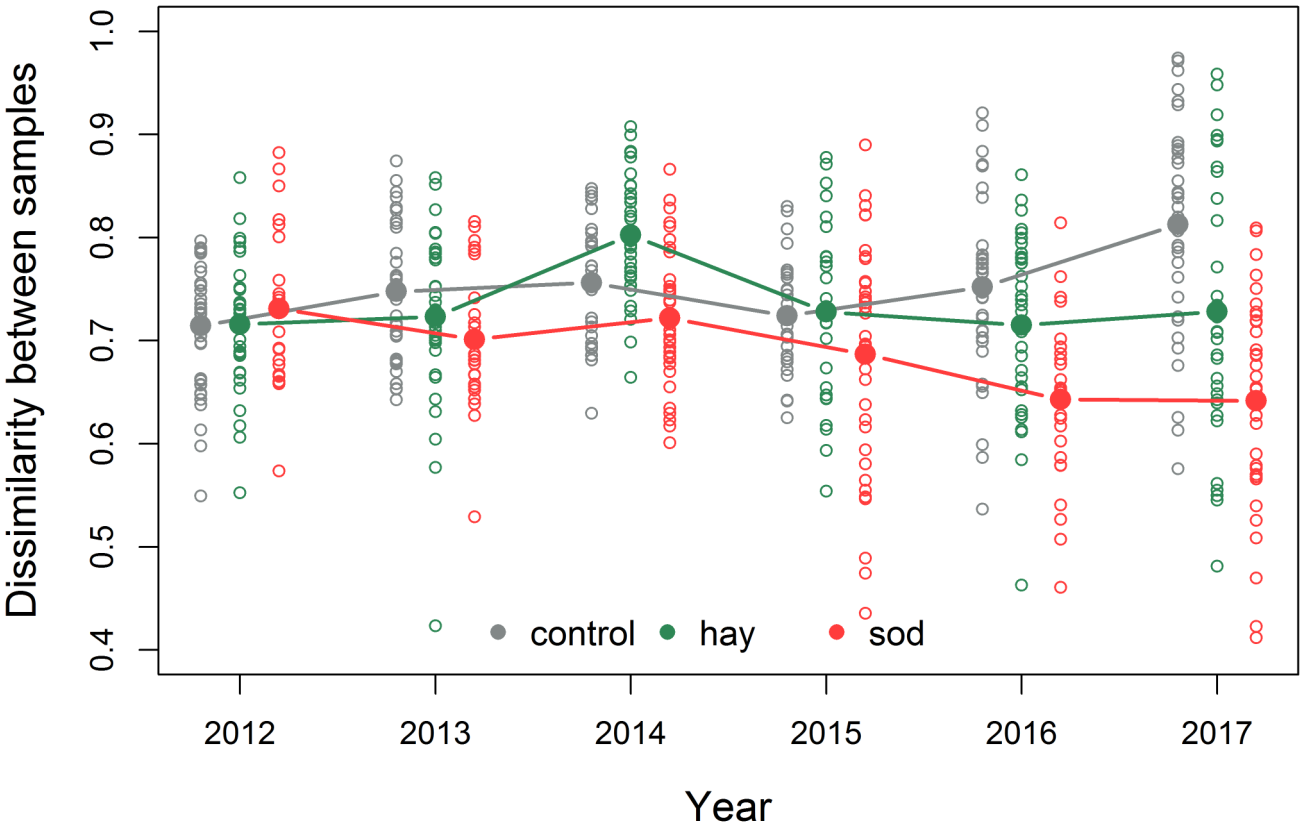


Figure 3

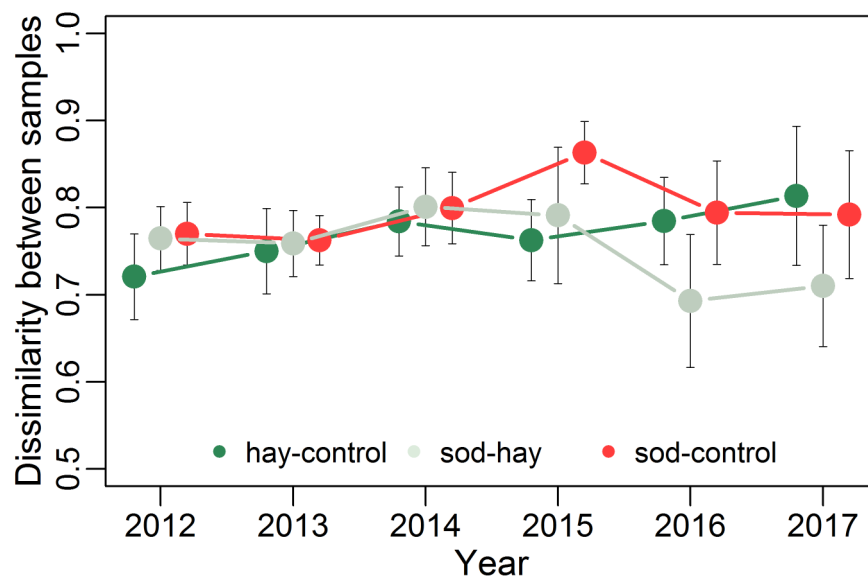


Figure 4

